

Cell Fusion Induced by Herpes Simplex Virus Glycoproteins gB, gD, and gH-gL Requires a gD Receptor but Not Necessarily Heparan Sulfate

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To characterize cellular factors required for herpes simplex virus type 1 (HSV-1)-induced cell fusion, we used an efficient and quantitative assay relying on expression of HSV-1 glycoproteins in transfected cells. We showed the following: (1) Cell fusion depended not only on expression of four viral glycoproteins (gB, gD, and gH-gL), as previously shown, but also on expression of cell surface entry receptors specific for gD. (2) Cell fusion required expression of all four glycoproteins in the same cell. (3) Heparan sulfate was not required for cell fusion. (4) Coexpression of receptor with the four glycoproteins in the same cell reduced fusion activity, indicating that interaction of gD and receptor can limit polykaryocyte formation. Overall, the viral and cellular determinants of HSV-1-induced cell fusion are similar to those for viral entry, except that HSV-1 entry is significantly enhanced by binding of virus to cell surface heparan sulfate. © 2001 Academic Press

INTRODUCTION

Multinucleated giant cells are a hallmark of herpes simplex virus type 1 (HSV-1) lesions *in vivo* (Spear, 1993a). In cultured cells, however, wild-type HSV-1 strains usually do not form polykaryocytes. Nonlethal mutations in one of several HSV-1 genes, including gB (UL27), gK (UL53), UL20, and UL24, can give rise to polykaryocyte-forming mutants *in vitro* (Baines *et al.*, 1991; Bond and Person, 1984; Bzik *et al.*, 1984; Cai *et al.*, 1988; Debroy *et al.*, 1985; DeLuca *et al.*, 1982; Dolter *et al.*, 1994; Engel *et al.*, 1993; Gage *et al.*, 1993; Hutchinson *et al.*, 1992b; Jacobson *et al.*, 1989; Pogue-Geile and Spear, 1987; Ruyechan *et al.*, 1979; Sanders *et al.*, 1982; Weise *et al.*, 1987). In addition to a syncytial mutation, HSV-1-induced polykaryocyte formation requires the expression of several viral glycoproteins, including gB, gD (US6), gH (UL22), and gL (UL1), the latter two of which are often designated as gH-gL because they are heterodimers (Hutchinson *et al.*, 1992a). The evidence is twofold. First, viral mutants that fail to express any one of these four glycoproteins will not form polykaryocytes (Cai *et al.*, 1988; Davis-Poynter *et al.*, 1994; Ligas and Johnson, 1988; Roop *et al.*, 1993). Second, expression of HSV-1 gB, gD, and gH-gL is necessary and sufficient to induce cell fusion in a Cos-Vero cell transfection system (Turner *et al.*, 1998). Similarly, expression of HSV-2 gB,

gD, and gH-gL in various cell lines results in polykaryocyte formation (Muggeridge, 2000).

Additional viral proteins can influence the ability of HSV-1 to form polykaryocytes in cultured cells. For example, studies with viral mutants deleted for specific genes suggest that gE (US8), gI (US7), gM (UL10), and UL45 can enhance syncytium formation (Davis-Poynter *et al.*, 1994; Haanes *et al.*, 1994; Visalli and Brandt, 1991), whereas gC (UL44) can inhibit fusion of at least some cell types (Manservigi *et al.*, 1977; Novotny *et al.*, 1996; Pertel and Spear, 1996). This latter observation may partially explain the absence of gC expression in many syncytial mutants (Spear, 1993a). In all these above-cited studies, it was not determined whether specific cell receptors for viral ligands were required for polykaryocyte formation or whether the requirements for various viral proteins in different cell types could be dependent on which cell receptors were expressed, assuming receptors are necessary.

HSV-1 entry is related to HSV-1-induced cell fusion, as penetration proceeds by fusion of the viral envelope with the cell membrane and requires many of the same viral glycoproteins. Initial binding of HSV-1 occurs through an interaction of gC (UL44) or gB with cell surface glycosaminoglycans (GAGs), primarily heparan sulfate (Herold *et al.*, 1991, 1994; Gruenheid *et al.*, 1993; Shieh *et al.*, 1992; Vaheri and Cantell, 1963; WuDunn and Spear, 1989). Subsequent fusion between the virion envelope and cell membrane requires gB, gD, and gH-gL, and possibly the tegument protein UL25 (Addison *et al.*, 1984; Cai *et al.*, 1988; Forrester *et al.*, 1992; Ligas and Johnson, 1988; Roop *et al.*, 1993). Penetration of virus requires the

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interaction of gD with specific cell surface receptors (Cocchi *et al.*, 1998; Geraghty *et al.*, 1998; Montgomery *et al.*, 1996; Warner *et al.*, 1998) or heparan sulfate modified by 3-O-sulfotransferases (3-OST) (Shukla *et al.*, 1999a).

Proteins from two families can function as gD receptors and mediate HSV-1 entry. Herpesvirus entry mediator (HVEM), a member of the tumor necrosis factor (TNF) receptor family, can mediate entry of wild-type HSV-1 into Chinese hamster ovary (CHO) cells, a cell line normally resistant to HSV-1 infection (Montgomery *et al.*, 1996). HVEM was also previously designated herpesvirus entry protein A (HveA) (Warner *et al.*, 1998) but will be called HVEM here because most publications use this name. Additional entry receptors are members of the immunoglobulin superfamily, including proteins originally designated poliovirus receptor-related protein 1 (Prr1) or HveC (Geraghty *et al.*, 1998) and poliovirus receptor-related protein 2 (Prr2) or HveB (Warner *et al.*, 1998). HveC and HveB were recently renamed nectin-1 and nectin-2, respectively, on the basis of function in cells (Spear *et al.*, 2000). Nectin-1 and nectin-2 are cell adhesion molecules that localize to cadherin-based cell junctions (Aoki *et al.*, 1997; Lopez *et al.*, 1998; Satoh-Horikawa *et al.*, 2000; Takahashi *et al.*, 1999).

As mentioned above, the viral ligand for HVEM, nectin-1, and nectin-2 is gD (Cocchi *et al.*, 1998; Geraghty *et al.*, 2000; Krummenacher *et al.*, 1998; Nicola *et al.*, 1998; Whitbeck *et al.*, 1997). Single amino acid substitutions in gD (Rid mutations) can alter the ability of virus to interact with a specific receptor. For example, nectin-2 mediates entry of specific HSV-1 mutants that express Rid forms of gD (gD-Rid) but cannot mediate entry of wild-type HSV-1 strains (Lopez *et al.*, 2000; Warner *et al.*, 1998). In contrast, HVEM mediates entry of wild-type virus but not Rid mutants (Montgomery *et al.*, 1996). Nectin-1 can mediate entry of both wild-type HSV-1 strains and HSV-1 Rid mutants (Geraghty *et al.*, 1998; Menotti *et al.*, 2000).

The cellular factors required for HSV-1-induced cell fusion are less well defined. A requirement for heparan sulfate was suggested by findings that the syncytial mutant HSV-1(MP) could induce the fusion of wild-type CHO cells but not of mutant cells defective for heparan sulfate or all GAG biosynthesis (Shieh and Spear, 1994). Wild-type CHO cells are more susceptible to HSV-1(MP) entry than to most HSV-1 strains (Shieh *et al.*, 1992; Shieh and Spear, 1994), and it may be that HSV-1(MP) uses an unidentified hamster cell surface receptor that is dependent on synthesis of heparan sulfate. A different syncytial mutant, HSV-1(KOS)804, fails to induce the fusion of wild-type CHO cells (Terry-Allison *et al.*, 1998). However, CHO cells expressing HVEM, nectin-1, or nectin-2 can fuse when transfected with HSV-1(KOS)804 genomic DNA, depending on the form of gD expressed (Terry-Allison *et al.*, 1998, 2000). HSV-1(KOS)804, which expresses wild-type gD, can fuse CHO cells expressing HVEM or nectin-1 but not nectin-2. In contrast, HSV-1(KOS)804 recom-

binants expressing Rid1 gD can fuse CHO cells expressing nectin-1 or nectin-2 but not HVEM. In these studies it was noted that inhibition of GAG sulfation could partially inhibit cell fusion mediated by nectin-2 but not nectin-1 or HVEM. Because these studies were performed under conditions by which all viral genes could be expressed, it was unclear whether viral proteins in addition to gD might influence receptor requirements for cell fusion.

Here we describe a quantitative and efficient cell fusion assay based on the expression of a limited subset of viral glycoproteins and defined entry receptors. We demonstrate that cell fusion induced by HSV-1 glycoproteins required the expression of specific viral entry receptors but not heparan sulfate or chondroitin sulfate, at least not as coreceptors with HVEM, nectin-1, or nectin-2. In addition, all four viral glycoproteins (gB, gD, and gH-gL) were necessary and sufficient for cell fusion, regardless of which entry receptor was available. Cell fusion was observed only when all four viral glycoproteins were expressed in the same cell and when the form of gD expressed (wild-type or Rid) was compatible with the available receptor. Coexpression of an entry receptor with the four glycoproteins in the same cell reduced the efficiency of cell fusion. These results define a minimal set of requirements for HSV-induced cell fusion, with respect to viral glycoproteins and cell surface receptors, and indicate that an interference phenomenon is operative in cell fusion.

RESULTS

Cell fusion requires an entry receptor and four HSV-1 glycoproteins

Genes encoding the four HSV-1 glycoproteins, gB, gD, gH, and gL, were cloned into the plasmid pCAGGS/MCS, which permits expression of inserts under control of the chicken β -actin promoter and human cytomegalovirus (CMV) immediate-early (IE) enhancer (Niwa *et al.*, 1991). As shown in Fig. 1, transfection of the resulting four plasmids into cells susceptible to HSV-1 infection (Vero, HeLa, and PEAK cells) resulted in the formation of polykaryocytes. The polykaryocytes were larger and more numerous on PEAK cells than on the other cell types, perhaps reflecting relative efficiencies of transfection. CHO-K1 cells, which lack an entry receptor for wild-type HSV-1 and are not susceptible to infection, failed to form polykaryocytes (Fig. 1). This is despite the presence of all four glycoproteins on the cell surface, as shown in Fig. 2, regardless of whether each glycoprotein was expressed alone or in combination. Less cell surface expression of gB, gD, or gD-Rid was noted when all four glycoproteins were expressed together rather than alone. This may represent differences in expression, exposure on the cell surface, or ability to detect exposure. However, as shown below, CHO-K1 cells express-

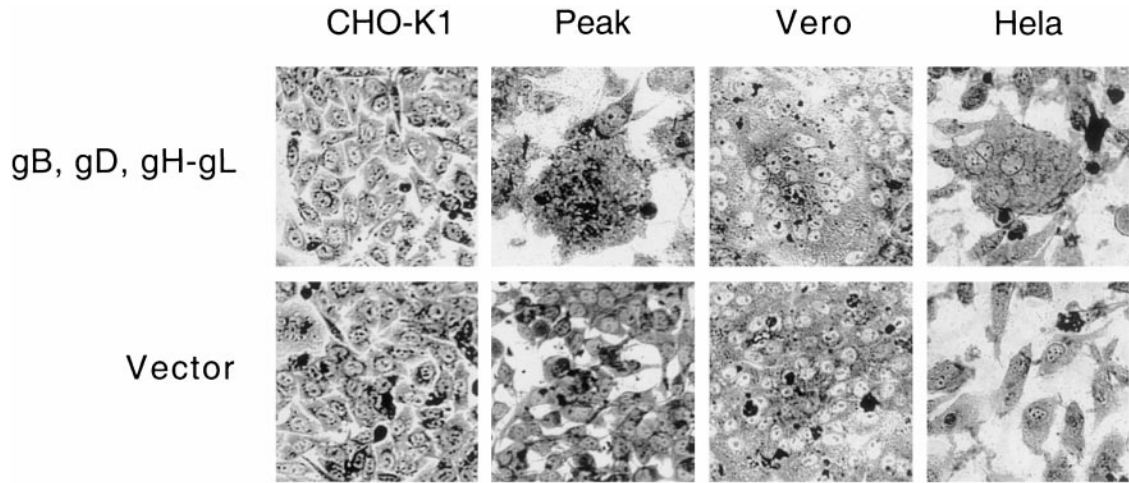


FIG. 1. HSV-1 glycoprotein-induced fusion of cell lines susceptible to viral infection. Cell lines susceptible (Vero, HeLa, and PEAK) and resistant (CHO-K1) to HSV-1 infection were transfected with plasmids expressing the indicated HSV-1 glycoproteins. After incubation for 24 h, cells were fixed and then stained with Giemsa. Shown are photographs of representative cell monolayers.

ing all four glycoproteins could induce polykaryocytes if mixed with cells expressing HSV-1 entry receptors.

These results confirm previous studies indicating that all four HSV-1 or HSV-2 glycoproteins (gB, gD, and gH-gL) are necessary to induce the fusion of transfected cells (Muggeridge, 2000; Turner *et al.*, 1998). Figure 1 shows that expression of these four glycoproteins alone is not sufficient because CHO-K1 cells did not fuse. We thus tested whether CHO-K1 cells expressing one of the known entry receptors could be induced to fuse by the four HSV-1 glycoproteins. Because HSV-1 entry mediated by HVEM, nectin-1, or nectin-2 depends on expression of an appropriate form of gD by virus, we also tested whether the ability of receptor-bearing cells to fuse depends on the form of gD expressed. To these ends, we mixed CHO-K1 cells expressing HSV-1 glycoproteins with CHO-K1 cells expressing herpesvirus entry receptors. In these and subsequent experiments, we defined effector cells as the cells expressing the HSV-1 glycoproteins and target cells as the cells to which we added the effector cells.

As shown in Fig. 3, effector cells expressing gB, gD, and gH-gL could form polykaryocytes with target cells expressing HVEM or nectin-1 (CHO-HVEM or CHO-nectin-1). We observed no polykaryocytes when we mixed effector cells with CHO-nectin-2 cells. In contrast, effector cells expressing gB, gD-Rid, and gH-gL could form polykaryocytes with CHO-nectin-1 and CHO-nectin-2 cells but not with CHO-HVEM cells. As expected, the effector cells failed to induce fusion with CHO-K1 cells expressing no receptor.

To quantify HSV-1 glycoprotein-mediated cell fusion, we used a luciferase reporter gene activation assay (Okuma *et al.*, 1999). We expressed T7 RNA polymerase and the HSV-1 glycoproteins of interest in effector cells and examined the activation of a luciferase reporter gene

under control of the T7 promoter in target cells after cocultivating the two cell populations. As shown in Table 1, a significant enhancement in luciferase activity was detected only under the experimental conditions that resulted in polykaryocyte formation, as shown in Fig. 3. In addition, expression of fewer than all four HSV-1 glycoproteins in the effector cells resulted in no significant luciferase activity (Table 1) and no polykaryocyte formation (data not shown). This finding was true for all three target cell lines tested, CHO-HVEM, CHO-nectin-2, and CHO-nectin-1.

Thus, the induction of cell fusion requires, at a minimum, expression of four viral glycoproteins (gB, gD, and gH-gL) and a herpesvirus entry receptor, such as the gD-receptors HVEM, nectin-1, or nectin-2.

Kinetics of HSV-1 glycoprotein-mediated cell fusion

To study the kinetics of fusion, we followed expression of luciferase activity and polykaryocyte formation over time. As shown in Fig. 4, maximal luciferase activity occurred approximately 18 h after cocultivating effector and susceptible target cells. Polykaryocytes were first visualized approximately 12 h after cocultivating effector and target cells, but were most prominent between 24 and 36 h (Fig. 5). Figure 5 shows results obtained using CHO-nectin-1 target cells but similar results were seen with the other susceptible target cells (data not shown). In general, the number and size of visible polykaryocytes correlated with the level of luciferase activity until 18 h.

In the time-course experiments, luciferase activity consistently decreased after 18 h, while the number of polykaryocytes remained the same or even increased. The decline in luciferase activity most likely resulted from loss of functional plasmids along with rapid deg-

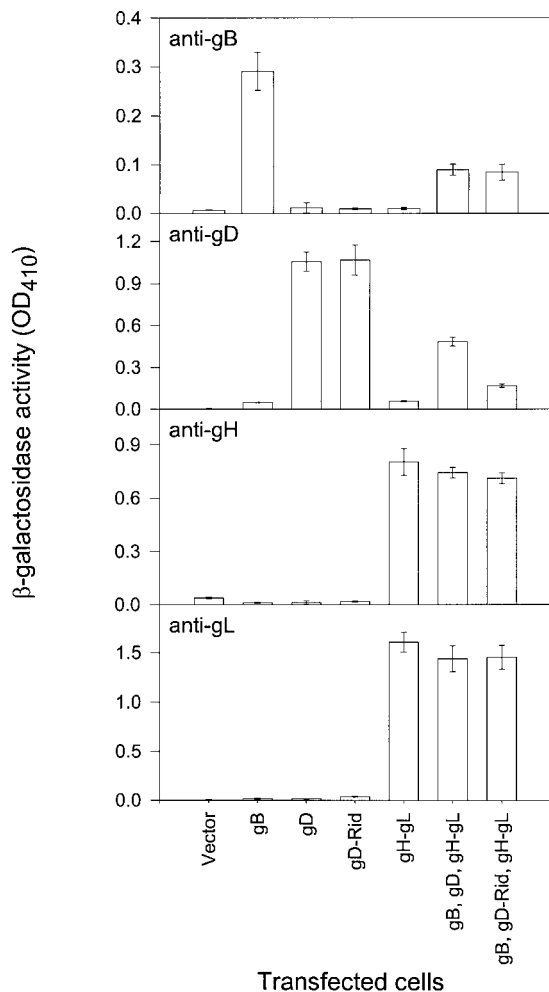


FIG. 2. Cell surface expression of HSV-1 glycoproteins detected by binding of specific antibodies. CHO-K1 cells were transfected with plasmids expressing the indicated HSV-1 glycoproteins. After replating onto 96-well dishes and overnight incubation, cells were incubated with primary antibody diluted in PBS with 3% BSA. Cells were subsequently fixed and then sequentially incubated with species-specific biotinylated anti-IgG conjugate and streptavidin- β -galactosidase conjugate. After addition of ONPG substrate, OD readings were obtained. Shown are the mean values and standard deviations for five replicate samples.

radation of luciferase mRNA and protein (Thompson *et al.*, 1991). Viral glycoproteins do not turn over rapidly and, despite the loss of the plasmids, would be available to mediate further cell fusion.

In all the experiments presented here, the effector and target cells were mixed and cocultivated 12 h after initiating DNA transfection. We obtained similar time courses when the cells were mixed and cocultivated 24 h after initiating transfection. In both cases, maximal luciferase activity was noted 18 h after cocultivating the effector and target cells. However, the level of luciferase expression was lower for cells mixed at 24 h after transfection (data not shown).

Cell surface glycosaminoglycans are not required for cell fusion

It was shown previously that undersulfation of cell surface GAGs partially reduces the level of HSV-1-induced fusion of cells expressing nectin-2 but not nectin-1 or HVEM (Terry-Allison *et al.*, 2000). In this study, however, viral genomes were transfected into cells to induce cell fusion, permitting expression of all viral proteins, some of which might have influenced the results. Moreover, fusion was assessed by microscopic analysis and was difficult to quantitate. To investigate whether cell surface GAGs are required for glycoprotein-induced cell fusion, we used the luciferase assay to quantitate the amount of fusion induced by HSV-1 glycoproteins in GAG-deficient cells compared with that of wild-type cells. We used CHO-K1 cells and the xylosyltransferase-deficient cell line CHO-745, which does not produce either heparan or chondroitin sulfate (Esko *et al.*, 1985). CHO-K1 and CHO-745 cells were transfected with plasmids expressing the desired receptors along with the luciferase reporter plasmid to serve as target cells. The same two cell lines were transfected with all four glycoproteins and T7 RNA polymerase to serve as effector cells. CHO-K1 effectors were mixed with CHO-K1 targets and CHO-745 effectors were mixed with CHO-745 targets, respectively, and the induction of luciferase activity was quantitated.

As shown in Fig. 6, cocultivation of mutant effector and target cells deficient for both heparan sulfate and chondroitin sulfate, or of wild-type cells, resulted in induction of luciferase activity, provided the target cells expressed an appropriate gD-specific entry receptor. Consistent with our previous results, expression of wild-type gD was required for efficient fusion with target cells that expressed HVEM or nectin-1, whereas gD-Rid was required for target cells that expressed nectin-2 or nectin-1, regardless of whether the cells were wild-type or GAG-negative. Microscopic analysis of the cell mixtures in which luciferase activity was significantly enhanced revealed the presence of polykaryocytes in both the wild-type and GAG-negative cells (data not shown). Similar results were obtained from three additional experiments (data not shown). In all these experiments, luciferase activities observed with the GAG-negative cells were either comparable to, or slightly lower than, those observed with the wild-type CHO cells. Because, in the two different cell types, it is difficult to control for the number of transfected cells, the levels of transiently expressed proteins and replating efficiencies, we cannot exclude the possibility that GAGs may influence the level of cell fusion. We can conclude, however, that expression of heparan sulfate or chondroitin sulfate is not required for HSV-1 glycoprotein-induced cell fusion dependent on HVEM, nectin-1, or nectin-2.

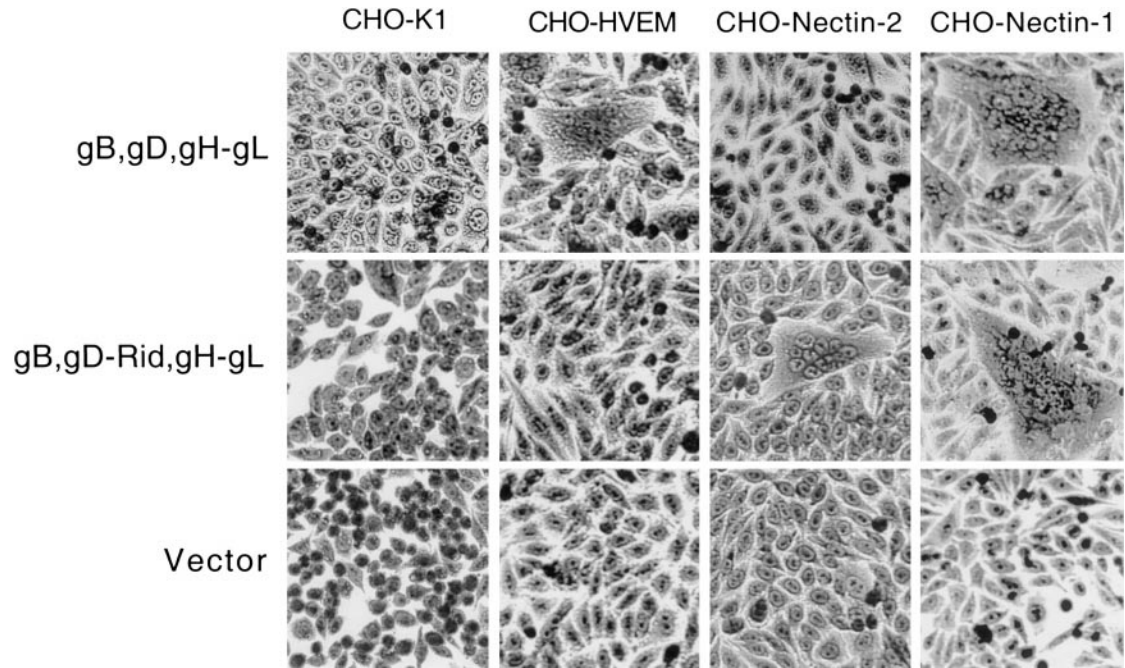


FIG. 3. HSV-1 glycoprotein-induced cell fusion depends on both the entry receptor and the form of gD expressed. CHO-K1 effector cells transfected with plasmids expressing the indicated HSV-1 glycoproteins or with control vector were cocultivated with CHO-K1, CHO-HVEM, CHO-nectin-2, or CHO-nectin-1 target cells. After incubation for 24 h, the cells were fixed and then stained with Giemsa. Shown are photographs of representative cell monolayers.

Efficient fusion requires that HSV-1 gB, gD, and gH-gL are expressed in the same cell

To determine whether cell fusion requires expression of gB, gD, and gH-gL in the same cell, we transfected CHO-K1 and CHO-nectin-1 cells with different combinations of HSV-1 glycoproteins and then cocultivated the cells. Two findings are evident from the results of these experiments (Table 2): (1) Polykaryocytes were visualized only when all four glycoproteins were transfected together into the same cell, either CHO-K1 or CHO-nectin-1

cells. In both cases, there were receptor-bearing cells with which glycoprotein-expressing cells could fuse. (2) Significant luciferase activity was detected only when all four glycoproteins were transfected into the CHO-K1 cells and then mixed with receptor-bearing cells. In this situation, the CHO-K1 cells expressing the glycoproteins and T7 RNA polymerase could fuse with receptor-bearing CHO-nectin-1 cells and induce luciferase activity. However, when CHO-nectin-1 cells were transfected with the glycoproteins, these cells could fuse only with

TABLE 1

Viral Glycoproteins and Receptors Required for Cell Fusion Quantitated by Luciferase Activity

Glycoproteins expressed in CHO-K1 effector cells	Luciferase activity (RLU) induced in target cells			
	CHO-K1	CHO-HVEM	CHO-nectin-2	CHO-nectin-1
Vector	5.8 ± 1.2	2.2 ± 0.3	3.7 ± 0.9	4.1 ± 0.4
gB, gD	1.3 ± 0.2	0.8 ± 0.1	1.3 ± 1.1	1.7 ± 0.3
gB, gD-Rid	3.3 ± 1.1	1.1 ± 0.4	1.4 ± 0.4	1.3 ± 0.2
gB, gH, gL	3.1 ± 1.2	2.8 ± 0.5	3.4 ± 0.7	3.6 ± 1.5
gD, gH, gL	0.8 ± 0.1	1.0 ± 0.3	1.6 ± 0.6	3.5 ± 1.4
gD-Rid, gH, gL	1.9 ± 0.1	1.0 ± 0.3	2.0 ± 1.1	2.9 ± 0.5
gB, gD, gH, gL	1.2 ± 0.2	29 ± 8.2	0.6 ± 0.3	130 ± 43
gB, gD-Rid, gH, gL	4.5 ± 1.5	2.7 ± 0.9	140 ± 82	1100 ± 90

Note. CHO-K1 effector cells were transfected with plasmids expressing the indicated HSV-1 glycoproteins and T7 RNA polymerase. CHO-K1, CHO-HVEM, CHO-nectin-2, and CHO-nectin-1 target cells were transfected with luciferase reporter plasmid. Luciferase activity was measured 18 hours after cocultivating the effector and target cells. Shown are the mean RLU values and standard deviations from one experiment performed in triplicate. An additional experiment yielded similar results (data not shown).

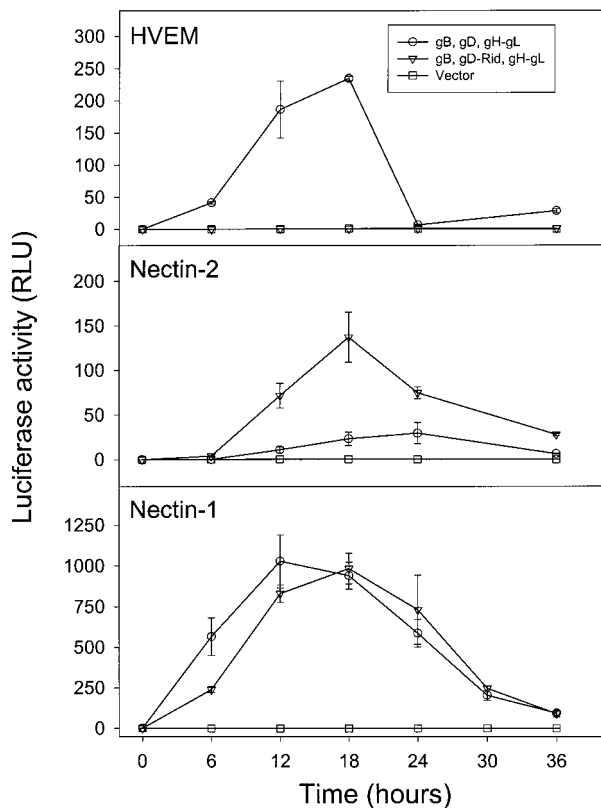


FIG. 4. Kinetics of cell fusion as measured by luciferase production. Luciferase activity was measured at the indicated times after cocultivating effector and target cells. CHO-K1 effector cells were transfected with plasmids expressing the indicated HSV-1 glycoproteins or control vector and T7 RNA polymerase. CHO-HVEM, CHO-nectin-2, and CHO-nectin-1 target cells were transfected with luciferase reporter plasmid. Two separate experiments each in duplicate were performed. Shown are mean relative luminosity unit (RLU) values and corresponding ranges.

other receptor-bearing cells but not with CHO-K1 cells expressing T7 RNA polymerase. Cells that did not express either glycoproteins or receptor were not incorporated into polykaryocytes as innocent bystanders.

Fusion can be inhibited by expressing receptor and HSV-1 glycoproteins in the same cell

Although we noted efficient polykaryocyte formation when receptor-bearing cells were transfected with all four HSV-1 glycoproteins, many cells did not fuse and the maximal size of the polykaryocytes was limited. Also, when glycoprotein-expressing effector cells fuse with receptor-bearing target cells, both viral glycoproteins and receptor are now coexpressed in the resulting polykaryocyte. Given that coexpression of gD with nectin-1 was previously shown to result in physical interactions between the two that correlate with loss of receptor availability for viral entry (Geraghty *et al.*, 2000), coexpression of gD and receptor within the same cell or polykaryocyte could result in loss of gD availability for initiation or enlargement of polykaryocytes.

To test this possibility, we transfected effector cells with the four required HSV-1 glycoproteins along with different entry receptors before cocultivating the cells with receptor-bearing target cells. In these experiments, we used combinations of glycoproteins and entry receptors that we previously showed could mediate cell fusion. Thus, CHO-K1 cells were transfected with plasmids expressing HSV-1 gB, gD, and gH-gL, and also with a plasmid expressing HVEM or nectin-1 or empty vector, and were then cocultivated with CHO-HVEM or CHO-nectin-1 target cells, respectively. Similarly, CHO-K1 cells were transfected with plasmids expressing gB, Rid-gD, and gH-gL, and also with a plasmid expressing nectin-2 or nectin-1 or empty vector, and then cocultivated with CHO-nectin-2 or CHO-nectin-1 target cells, respectively. As shown in Fig. 7, coexpression of HVEM or nectin-1 with gB, gD, and gH-gL reduced the induction of luciferase activity after cocultivation with the appropriate target cells to approximately 42 and 9% of the control levels, respectively (Fig. 7, left panel). Coexpression of nectin-2 and nectin-1 with gB, Rid-gD, and gH-gL reduced luciferase activity to approximately 13 and 2% of the control values, respectively (Fig. 7, right panel). Thus, cells expressing both receptor and HSV-1 glycoproteins fuse less efficiently with susceptible cells. This most likely results from interactions between gD and receptor in the effector cells that block gD binding to receptor on target cells.

DISCUSSION

We utilized a quantitative and efficient cell fusion assay to identify viral and cell surface requirements for cell fusion induced by HSV-1. We showed that four viral glycoproteins (gB, gD, and gH-gL) are necessary for cell fusion, confirming results obtained by others for HSV-1 or HSV-2 glycoproteins expressed in various primate cell types (Muggeridge, 2000; Turner *et al.*, 1998). In these previous studies, the potential requirement for cell surface receptors was not directly addressed, although soluble gD that can bind HSV entry receptors was shown to block HSV-2-induced cell fusion (Muggeridge, 2000). Here we show that expression of the four HSV-1 glycoproteins is not sufficient but that cell fusion also requires the expression of an HSV-1 entry receptor in the target cells. It was reported that an anti-nectin-1 antibody capable of blocking viral entry failed to block cell fusion induced by two syncytial strains of HSV-1 (Cocchi *et al.*, 2000). This result is not necessarily inconsistent with our conclusion that an entry receptor is required for cell fusion because entry receptors participating in cell fusion may not have been accessible to the antibody used or entry receptors other than nectin-1 may have been used. One of the strains used in this study, HSV-1(MP), was previously shown to infect cells devoid of all the known entry receptors (Montgomery *et al.*, 1996).

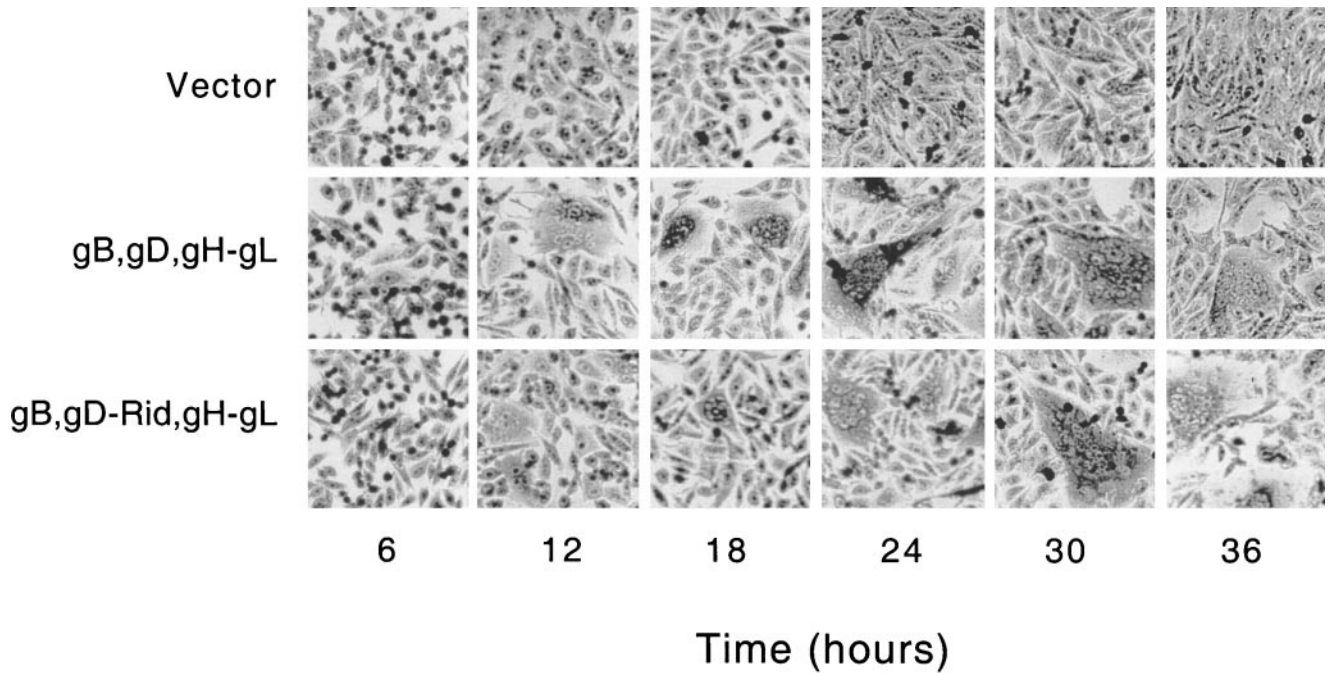


FIG. 5. Kinetics of cell fusion as assessed microscopically. At the indicated times, duplicate plates from the experiment shown in Fig. 4 were fixed and stained with Giemsa. Shown are photographs of representative cell monolayers from the cocultivation of effector cells and CHO-nectin-1 target cells.

The entry receptors demonstrated here to provide receptors for cell fusion were HVEM, nectin-1, and nectin-2. Each of these cell surface receptors binds to either wild-type gD or gD-Rid and mediates fusion in accordance with the form of gD expressed and recognized. Cell surface heparan sulfate or other GAGs were not required as coreceptors for cell fusion when the entry receptors expressed were HVEM, nectin-1, or nectin-2.

Thus, the viral and cellular determinants of HSV-1-induced cell fusion are similar to those required for viral entry (Spear *et al.*, 2000), except that entry is significantly enhanced by the presence of cell surface heparan sulfate, whereas cell fusion did not require expression of heparan sulfate or chondroitin sulfate. A likely explanation for this apparent difference in requirements is as follows. Although binding of virus to cells may be largely dependent on the binding of virus to heparan sulfate and subsequent virus-cell fusion depends on prior binding of virus, cocultivated cells can have close contacts mediated by cell factors other than heparan sulfate.

Results presented elsewhere also highlight the requirement for HSV-1 entry receptors in HSV-1-induced cell fusion and demonstrate relatively little effect on cell fusion of altering GAG structure by use of sulfate inhibitors (Terry-Allison *et al.*, 1998, 2000). However, these results were obtained by use of transfected viral genomes rather than plasmids expressing individual viral glycoproteins, so that effects of other viral gene products on the results could not be ruled out. For example, deletion of the gene for gE, gI, gM, or UL45 was previ-

ously reported to abrogate cell fusion induced by syncytial strains of HSV-1 (Davis-Poynter *et al.*, 1994; Haanes *et al.*, 1994; Visalli and Brandt, 1991), while expression of gC may inhibit the fusion of at least some cell types (Manservigi *et al.*, 1977; Novotny *et al.*, 1996; Pertel and Spear, 1996). The cell fusion assay described here should be very useful for assessing the ability of these other viral proteins to influence fusion induced by the essential glycoproteins interacting with defined cell surface receptors.

Although we found, using HSV-1(KOS) glycoproteins, that GAGs were not necessary for cell fusion dependent on HVEM, nectin-1, or nectin-2, this finding may not be generalizable to other HSV strains or to other entry receptors. Polykaryocyte formation by the syncytial strain HSV-1(MP) was shown to depend on the expression of GAGs in CHO cells (Shieh and Spear, 1994). Possibly HSV-1(MP) can use as an entry and fusion receptor an unidentified hamster cell surface molecule that is dependent on expression of heparan sulfate. It should be noted that a nonprotein entry receptor was identified for HSV-1, namely sites in heparan sulfate generated by the action of specific 3-*O*-sulfotransferases (Shukla *et al.*, 1999a). It seems reasonable to predict that HSV-1 glycoproteins could induce the fusion of cells expressing this modified form of heparan sulfate as the only entry receptor, and that this cell fusion would be totally dependent on the expression of heparan sulfate and the appropriate 3-*O*-sulfotransferase. Although this enzyme is not normally expressed in CHO cells, it may be that HSV-1(MP) can

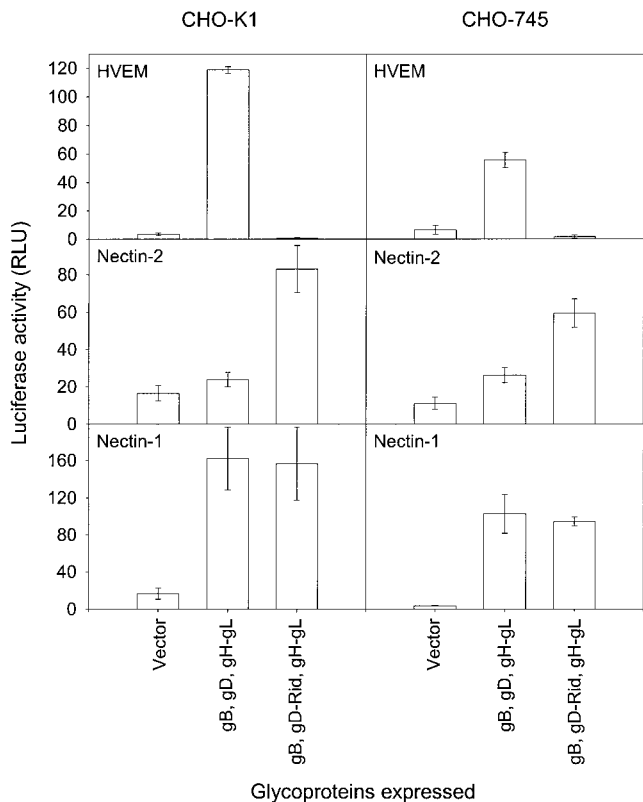


FIG. 6. Cell surface expression of heparan sulfate and chondroitin sulfate are not required for cell fusion. Effector cells were transfected with plasmids expressing HSV-1 gB, gD, and gH-gL or with control vector and T7 RNA polymerase. Target cells were transfected with luciferase reporter plasmid and plasmids expressing HVEM, nectin-2, or nectin-1. Both the effector and target cells were CHO-K1 cells in the left panel and CHO-745 cells in the right panel. Luciferase activity was measured 18 h after cocultivating the effector and target cells. Shown are the mean values and standard deviations from one experiment performed in triplicate. Similar results were obtained in three additional experiments (data not shown).

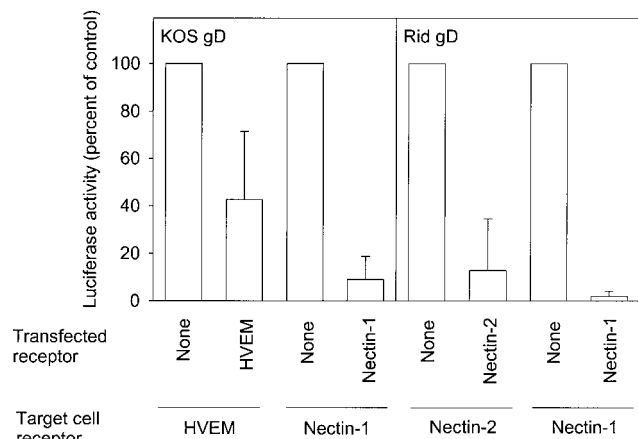


FIG. 7. Entry receptors coexpressed with HSV-1 glycoproteins in the effector cells inhibit cell fusion. In addition to all four HSV-1 glycoproteins and T7 RNA polymerase, CHO-K1 effector cells were transfected with either vector alone or plasmid expressing the entry receptor of interest. CHO-HVEM, CHO-nectin-1, and CHO-nectin-2 target cells were transfected with luciferase reporter plasmid. Luciferase activity was measured 18 h after cocultivating the effector and target cells. Results are presented as the luciferase activity obtained with effector cells coexpressing receptor as a percentage of the activity obtained with effector cells not expressing receptor. The panel on the left shows results for effector cells transfected with gB, gD, and gH-gL and cocultivated with CHO-HVEM or CHO-nectin-1 target cells. The panel on the right shows results for effector cells transfected with gB, Rid-gD, and gH-gL and cocultivated with CHO-nectin-2 and CHO-nectin-1 target cells. In all cases, the receptor transfected into the effector cells was the same as the receptor expressed in the target cells. Shown are the mean values and 95% confidence intervals from one experiment performed in triplicate. Similar results were obtained in one additional experiment (data not shown).

utilize heparan sulfate with the normal CHO pattern of sulfation as an entry and cell fusion receptor.

Recent results obtained with pseudorabies virus (PRV), a related alphaherpesvirus, indicate that the PRV

TABLE 2

Cell Fusion Requires That All Four HSV-1 Glycoproteins Are Expressed in the Same Cell

Glycoprotein expressed		Luciferase activity (RLU)	Visible polykaryocytes
CHO-K1 cells (T7 RNA polymerase)	CHO-nectin-1 cells (luciferase reporter plasmid)		
gB, gD, gH, gL	Vector	220 ± 14	Yes
gB, gD	gH, gL	0.5 ± 0.3	No
gD, gH, gL	gB	0.6 ± 0.2	No
gD	gB, gH, gL	1.2 ± 0.1	No
gB	gD, gH, gL	0.2 ± 0.0	No
gB, gH, gL	gD	0.7 ± 0.2	No
gH, gL	gB, gD	0.8 ± 0.1	No
Vector	gB, gD, gH, gL	5.0 ± 0.8	Yes

Note. CHO-K1 cells and CHO-nectin-1 cells were transfected with vectors expressing the indicated HSV-1 glycoproteins. CHO-K1 cells were also transfected with T7 RNA polymerase, while CHO-nectin-1 cells were transfected with luciferase reporter plasmid. Luciferase activity was measured 18 hours after cocultivating CHO-K1 and CHO-nectin-1 cells. Shown are mean RLU values and standard deviations from one experiment performed in triplicate. Two additional experiments yielded similar results (data not shown).

requirements for cell fusion differ from those for HSV. It was shown that transfection of plasmids expressing PRV gB and gH-gL was sufficient to induce the fusion of rabbit kidney cells (Klupp *et al.*, 2000). This finding is in keeping with previous studies showing that, in contrast to HSV-1, PRV can form plaques in the absence of PRV gD (Peeters *et al.*, 1992; Rauh and Mettenleiter, 1991). However, PRV and HSV-1 are alike in that (1) gB, gD, and gH-gL are all required for virion infectivity and viral penetration (Mettenleiter, 2000; Spear, 1993b) and (2) nectin-1 and nectin-2 bind to both HSV-1 and PRV gD (Geraghty *et al.*, 2000; Krummenacher *et al.*, 1998) and can serve as entry receptors for both viruses (Geraghty *et al.*, 1998; Shukla *et al.*, 1999b, 2000; Warner *et al.*, 1998). Interestingly, gD-negative mutants of PRV are noninfectious but can acquire secondary mutations during forced passage that permit the production of weakly infectious virus in the absence of gD (Schmidt *et al.*, 1997). Taken together, these findings suggest that, even for HSV, gD may not be an essential component of the viral membrane-fusing machinery but, through binding to a receptor, may serve as the usual trigger for activating membrane fusion. In the case of PRV, and perhaps also HSV, there may be additional receptors capable of interacting with gB or gH-gL to induce membrane fusion. The cell fusion assay described here should facilitate further definition of the variables that can influence alphaherpesvirus-induced cell fusion.

MATERIALS AND METHODS

Cells

J. D. Esko (University of California, San Diego) provided wild-type CHO-K1 cells and a mutant CHO cell line, pgsA-745 (designated here as CHO-745). CHO-745 cells are xylosyltransferase-deficient and do not produce either heparan sulfate or chondroitin sulfate (Esko *et al.*, 1985). HVEM (CHO-HveA35), nectin-2 (CHO-Prr2), and nectin-1 (CHO-Prr1) expressing CHO cells were previously described (Geraghty *et al.*, 1998; Montgomery *et al.*, 1996; Warner *et al.*, 1998) and are designated here as CHO-HVEM, CHO-nectin-2, and CHO-nectin-1, respectively. All CHO cell lines were passaged in Ham's F12 medium (Gibco BRL, Gaithersburg, MD) supplemented with 10% fetal bovine serum (FBS) and Geneticin (400 $\mu\text{g}/\text{ml}$; Gibco BRL). Vero cells are an adult African green monkey cell line obtained from American Tissue Culture Collection (ATCC, Rockville, MD), and were passaged in 199 medium supplemented with 5% FBS. HeLa cells are derived from a cervical epitheloid carcinoma (ATCC). PEAK cells are human embryonic kidney cells that express the large T antigen and are modified to enhance transfection efficiency (Edge BioSystems, Gaithersburg, MD). HeLa and PEAK cells were passaged in Dulbecco's modified Eagle's medium supplemented with 10% FBS.

Cell enzyme-linked immunosorbent assay (CELISA)

Subconfluent CHO-K1 cells were transfected with plasmids expressing HSV-1 glycoproteins using lipofectamine reagent (Gibco BRL). Individual wells were transfected with 0.4 μg of each expression vector (0.8 μg in the case of gB), keeping the total amount of DNA added at 2.4 μg per well by the addition of empty vector DNA. After incubation at 37°C in 5% CO₂ for 8 h, the transfection mixture was removed and Ham's F12 containing 10% FBS was added. The cells were incubated for an additional 4 h before detaching with 0.25% trypsin and 1 mM tetrasodium ethylenediaminetetraacetic acid (EDTA; Gibco BRL) and replating onto 96-well plates. After incubation for 18 h, cells were washed with phosphate-buffered saline (PBS) and then incubated with primary antibody diluted in PBS containing 3% bovine serum albumin (BSA). Mouse monoclonal antibodies used included the anti-gH antibodies 52S and 53S (culture supernatant diluted 1:1), the anti-gL antibodies VIII87 and VIII200 (ascites fluid diluted 1:1000), and the anti-nectin-1 antibody XIV207 (undiluted culture supernatant). Rabbit polyclonal antibodies used included the anti-gB antibody R74, the anti-gD antibody R7, the anti-HVEM antibody R11874, and the anti-nectin-2 antibody R146. All rabbit antibodies were diluted 1:1000. Hybridoma cells producing 52S and 53S (HB8181 and HB8182, respectively) are from ATCC. All other antibodies were previously described (Herold *et al.*, 1994; Isola *et al.*, 1989; Montgomery *et al.*, 1996; Novotny *et al.*, 1996; Warner *et al.*, 1998), except XIV207 (R. J. Geraghty and P. G. Spear, unpublished data). Subsequently, cells were fixed with PBS containing 2% formaldehyde and 0.2% glutaraldehyde and incubated sequentially with species-specific biotinylated anti-IgG conjugate and streptavidin- β -galactosidase conjugate. After adding *o*-nitrophenol- β -D-glucopyranoside (ONPG) substrate (3 mg/ml in PBS; Sigma, St. Louis, MO), optical density (OD) readings at 410 nm were obtained by a plate spectrophotometer (SpectraMax 250, Molecular Devices, Sunnyvale, CA).

Construction of expression vectors

Y. Kawaoka (University of Wisconsin, Madison) provided the expression plasmid pCAGGS/MCS. This plasmid was constructed by inserting a polylinker into pCAGGS (Kobasa *et al.*, 1997). Proteins inserted into pCAGGS are expressed under control of the chicken β -actin promoter and CMV IE enhancer (Niwa *et al.*, 1991).

The HSV-1(KOS) glycoprotein open reading frames of interest were inserted into pCAGGS/MCS as follows. The *Bam*HI G fragment of genomic HSV-1(KOS) DNA, which encodes the gB open reading frame, was digested with *Kpn*I, inserted into the *Kpn*I site of pUC18, and screened for orientation, generating pDW20 (D. WuDunn and P. G. Spear, unpublished data). PCR mutagenesis was used to

generate an upstream *Bgl*II site and downstream *Mun*I site. For the *Bgl*II site, the two primer pairs consisted of sense primer 5'-GGCCCCGT**AGATCT**GCCATGCAC-CAGG-3' and antisense primer 5'-TGGTACGCGATCA-GAAAGCC-3' along with sense primer 5'-CCCTCTTT-GATCACTCCACC-3' and antisense primer 5'-TGCATG-GC**AGATCT**ACGGGGGCCCGTCG-3'. For the *Mun*I site, the two primer pairs consisted of sense primer 5'-CGACTTTGACGAGGCCAAGC-3' and antisense primer 5'-GGTTT**CAATTG**ACAACAAACCC-3' along with sense primer 5'-GTTGT**CAATTG**AAACCACGG-3' and antisense primer 5'-CCCAGTCACGACGTTGAAA-3'. The introduced restriction endonuclease recognition sequences are shown in bold. The Northwestern University Biotechnology Facility, the Great Lakes Regional Center for AIDS Research (CFAR), or Operon Technologies (Alameda, CA) constructed all primers used for cloning and sequencing. After removing an *Xba*I site in a noncoding region of the vector, the *Bgl*II and *Mun*I sites were converted into *Avr*II and *Xba*I sites, respectively, using linkers (*Avr*II linker: 5'-GCCTAGGC-3'; *Xba*I linker: 5'-GCTCTAGAGC-3'; New England Biolabs, Beverly, MA). Subsequently, the gB gene was excised with *Avr*II and *Xba*I, inserted into the *Nhe*I site of pCAGGS/MCS, and screened for orientation, generating pPEP98. Nucleotide sequencing (The Northwestern University Biotechnology Facility and CFAR) of the gB open reading frame revealed several polymorphisms previously described in other HSV-1 strains, including KOS. One additional polymorphism was noted at nucleotide position 1544 (G to A) that results in an arginine-to-histidine substitution. This polymorphism was in a region of the open reading frame outside the region that could have been altered by PCR mutagenesis. The gB nucleotide sequence was deposited with GenBank (accession number AF311740).

Construction of plasmids containing HSV-1(KOS) gD (pPEP26), HSV-1(KOS)Rid1 gD (pCJ1), and HSV-1(KOS) gH (pMN7) and gL (pMN49) were previously described (Geraghty *et al.*, 2000; Novotny, 1996; Novotny *et al.*, 1996; Pertel and Spear, 1997). These glycoproteins were subcloned into pCAGGS/MCS as follows. The gD gene was excised by digestion with *Eco*RI and *Sph*I and inserted between these sites of pCAGGS/MCS, generating pPEP99. The Rid1 gD gene was excised by sequential digestion with *Afl*III, large (Klenow) fragment of DNA polymerase I, and *Bam*HI, and subsequently inserted between the *Sma*I and *Bgl*II sites of pCAGGS/MCS, generating pPEP108. The gH gene was excised with *Sac*I and *Sph*I and also inserted between these sites, generating pPEP100. The gL gene was excised by digestion with *Eco*RI and *Nae*I and inserted between the *Eco*RI and *Sma*I sites of pCAGGS/MCS, generating pPEP101.

The plasmid pCAGT7 expresses T7 RNA polymerase using the chicken β -actin promoter and CMV IE enhancer (Okuma *et al.*, 1999). The plasmid pT7EMCLuc expresses the firefly luciferase gene using the T7 pro-

moter. Y. Matsuura (National Institute of Infectious Diseases, Tokyo, Japan) provided both plasmids. The plasmids expressing HVEM, nectin-2, and nectin-1 were described previously (Geraghty *et al.*, 1998; Montgomery *et al.*, 1996; Warner *et al.*, 1998).

Fusion assays

Cell fusion was visualized by microscopy following Giemsa staining or quantified using a luciferase reporter gene activation assay. Photographs of the cells were taken with an Olympus or Nikon digital camera. For the luciferase assay (Okuma *et al.*, 1999), subconfluent effector cells in six-well plates were transfected with the vectors expressing the glycoproteins of interest and T7 RNA polymerase. Target cells in six-well plates were transfected with a vector expressing luciferase under control of the T7 promoter. Individual wells were transfected with 0.4 μ g of each plasmid. The total amount of DNA transfected per well was kept constant at 2.4 μ g per well by adding vector DNA. Lipofectamine reagent in Opti-MEM was used for all transfections. After incubation at 37°C in 5% CO₂ for 8 h, the transfection mixture was removed, Ham's F12 containing 10% FBS was added, and the cells were incubated for an additional 4 h. The approximate cell density at this point was 2×10^4 /well. Cells were subsequently detached using trypsin-EDTA and washed with complete media. Effector and target cells were mixed in a 1:1 ratio and then replated onto 24-well dishes. Optimal luciferase expression occurred when effector and target cells were detached, mixed at a 1:1 ratio, and cocultivated 12 h after initiating the transfection (data not shown). Six to 36 h later, luciferase activity was quantified using the Luciferase Reporter Assay System (Promega, Madison, WI). Briefly, after washing with PBS, cells were lysed with passive lysis buffer, the supernatant was collected, and beetle luciferin substrate was added. Luminosity readings were obtained using a TD-20/20 luminometer (Turner Designs, Promega, Madison, WI). Supernatants were diluted 1:10 for all readings.

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